UNIVERSITY OF PENNSYLVANIA

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IN COLLABORATION WITH

PROFESSOR D. WRIGHT WILSON

A THESIS

IN PHYSIOLOGICAL CHEMISTRY

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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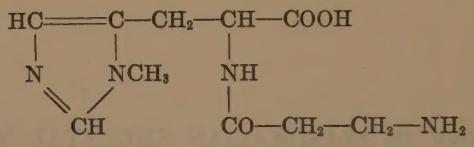
In the course of some experiments on the possible physiological significance of carnosine and related compounds we had occasion to study the content of the former in skeletal muscle from dogs. We expected a large yield of carnosine from this tissue as Krimberg (1) had mentioned a pure carnosine preparation from dog muscle, and as von Fürth and Schwarz (2) had reported "carnosine nitrogen" equivalent to some 4 gm. of free base per kilo of muscle. However, isolation procedures suitable for beef and other tissues yielded less than 35 mg. of base as crude copper salt per kilo of muscle. In one preparation we obtained evidence of the presence of anserine, a methyl carnosine recently discovered in goose muscle by Ackermann, Timpe, and Poller (3) and later isolated from several birds, reptiles (4), and fish (5). These investigators found none in beef (3), and later Keil (6) stated that carnosine but no anserine occurred in mammals, that anserine and possibly carnosine occurred in birds, and that both compounds occurred in reptiles. (". . . Bei Säugetieren Carnosin, aber kein Anserin; bei Vogelarten Anserin, vielleicht auch Carnosin; bei Reptilien mitunter Anserin zusammen mit Carnosin (Krokodil), mitunter aber auch nur Carnosin Python und Boa.")

Ackermann and his collaborators have isolated anserine as a copper salt from the lysine fraction of a mercuric sulfate-alcohol

precipitate from the aqueous extract of certain muscles (3). The base dried at 120°, analyzed as $C_{10}H_{16}N_4O_3$, contained one free amino group, one carboxyl group, and one methylimino group (7). Hydrolysis with barium hydroxide yielded *dl*-methylhistidine and β-alanine. Distillation with soda-lime in a stream of hydrogen yielded 1,5-dimethyliminazole identical with Pyman's synthetic product (6–8). Dinitrotolyl anserine on hydrolysis yielded dinitrotolyl-β-alanine (6). These data indicated that anserine is β-alanyl-*N*-methylhistidine, with the iminazole ring substituted in positions (1) and (5) as shown below.



The copper salt of anserine forms a deep blue solution in water and separates out at low temperatures as blue needles, m.p. 230–232°. On drying in air the blue form changes to a second modification, red-lilac in color, which may also be obtained from a hot concentrated alcoholic or aqueous solution. The red form turns blue on drying at 100° *in vacuo* but becomes red again on cooling in air. This characteristic behavior is valuable for identifying anserine.

The free base, usually prepared from the copper salt, crystallizes as colorless needles, is very soluble in water and difficultly soluble in absolute alcohol, melts at 238–239°, reacts as a base, and is dextrorotatory, $[\alpha]_D^{16} = +11.16°$ to 11.26°. From aqueous solution it is precipitated by phosphotungstic acid, mercuric salts with alcohol, flavianic acid, alcoholic picric acid, and tannic acid. Other derivatives which have been prepared are the nitrate, chloroplatinate, chloroaurate, and monopicrate (3).

Although this new compound has been assumed to be a characteristic extractive of muscles from lower vertebrates (6) we, on the basis of observations mentioned, undertook its preparation from dog muscle. A successful result prompted the examination of muscles from several species of mammals, of which the cat, rabbit, and rat also yielded anserine. No trace of the compound could be obtained in extracts from horse muscle.

Isolation Procedure

Preliminary to the study of anserine in mammalian muscle we repeated Ackermann's procedure on chicken muscle. The crude copper salt showed the characteristic properties described above and melted at about 224°.[1] Further purification and the preparation of derivatives was postponed for the time.

The procedure finally adopted for the present study differed only in minor details from that developed by Ackermann, Timpe, and Poller (3). Skeletal muscles dissected from a recently killed animal were ground in a meat chopper and thrice extracted with about 5 volumes of boiling water. The combined extracts were brought to the boiling point, acetic acid was added if necessary, and the protein coagulum removed on muslin. The filtrate was chilled, and after the removal of fat was evaporated over a free flame to about one-twentieth its volume; *i.e.*, to about three-fourths the volume of meat used. The warm viscous solution was slowly poured with stirring into 4 volumes of 95 per cent ethyl alcohol, left in the ice box overnight, and then filtered through coarse paper. The alcoholic filtrate was treated with mercuric sulfate reagent (250 gm. of $HgSO_4$ and 100 cc. of concentrated H_2SO_4 and water to make 1 liter) as long as a white flocculent precipitate formed. A slight excess of the mercury reagent was indicated by the formation of a yellow granular precipitate, probably a basic mercuric sulfate. The final solution usually contained 60 to 75 per cent alcohol by volume. After standing a few hours most of the alcoholic solution was decanted and the remainder was centrifugalized in 500 cc. bottles. After removing the supernatant fluid the mercury precipitate was thoroughly dispersed by suspending in water and decomposed with hydrogen sulfide—a process sometimes requiring 8 to 10 hours. The solution with washings from the mercuric sulfide was brought to a pH of 4 or 5 with barium hydroxide and filtered.

The filtrate with washings from the barium sulfate was fractionated by the Kossel-Kutscher procedure (9). Silver nitrate was added until a drop of the supernatant liquid removed to a

[1] Melting points reported in this paper are in reality "decomposition ranges." Thermometer readings corrected for stem exposure were checked against a total immersion thermometer certified by the Bureau of Standards.

watch-glass gave a dark brown precipitate with excess barium hydroxide. The precipitate of purine silver formed in the acid solution was removed by centrifugalizing and set aside. The histidine silver fraction, which precipitated on making the solution barely alkaline to litmus with barium hydroxide, was usually removed with the arginine silver fraction which came down on making the solution alkaline to tropeolin O with barium hydroxide. The strongly alkaline filtrate, called the lysine fraction, was neutralized to litmus with sulfuric acid and treated with hydrogen sulfide. The filtrate from the silver sulfide and barium sulfate, usually about 2 liters, was evaporated to 300 cc. on the water bath. About 100 cc. of mercuric sulfate reagent were then added and allowed to stand in the ice box overnight. The precipitate of barium sulfate with some organic material was rejected and the clear solution precipitated with alcohol and additional mercuric sulfate as described above. A second treatment with silver nitrate and barium hydroxide followed by one or two additional mercury-alcohol precipitations was sometimes necessary at this stage, especially if the alkaline solution reduced copper carbonate. Sulfate was removed quantitatively with barium hydroxide from the solution resulting from the decomposition of a final mercury precipitate. Freshly prepared moist copper carbonate added in excess to the final filtrate gave a deep blue solution which was filtered and evaporated on the steam bath. The typical red- or violet-colored copper anserine crystals were filtered on paper in a special centrifugal device.[2]

The copper salt thus prepared was frequently impure, as indicated by the melting point, nitrogen content, and color value (discussed below). For purification the salt was decomposed with hydrogen sulfide, taken through a silver nitrate-barium hydroxide treatment and reprecipitated with mercuric sulfate and alcohol. After decomposing the mercury precipitate with hydrogen sulfide and removing the sulfides and sulfuric acid, the solution was used to prepare the copper salt or was evaporated to 10 to 15 cc. under reduced pressure at 30–35°. For the free base this solution was evaporated further *in vacuo* over sulfuric acid until crystals ap-

[2] This consisted of a Gooch crucible fitted with a rubber collar and short tube permitting it to ride suspended in a centrifuge cup at 1500 R.P.M.

peared; for the nitrate, the solution was neutralized (to litmus) with nitric acid and evaporated until crystals appeared. When crystals of either free base or nitrate appeared 3 volumes of alcohol were added to increase the yield. Losses of anserine were considerable by using this procedure for purification, presumably because the silver-barium treatment precipitates some anserine from concentrated solution.

Color Values

Our anserine preparations from mammalian skeletal muscle were always contaminated with small amounts of carnosine or substances of similar nature. Since anserine gives no color with the diazo reagent the total chromogenic material present as impurity in any preparation can be estimated by treating a known amount with diazotized sulfanilic acid (10) and matching the resulting color against Hunter's dye standard (11). The amount of color may be calculated as per cent of that which would be produced by an equal weight of carnosine. We have called this value the color value of the preparation. The magnitude of the color value is an excellent index to the degree of contamination by carnosine and similarly reacting substances. A relatively high color value has been found on preparations giving a high nitrogen and low melting point. On treating such a preparation with silver nitrate and barium hydroxide the color value fell and the composition approached the theoretical; also, the color produced with the diazo reagent became more yellow. Although our best preparation gave a slight color with the diazo reagent our experience supports the statement of Ackerman, Timpe, and Poller (3) that repeated treatments with silver nitrate and barium hydroxide will reduce the color value to zero. Our best preparation of free base contained about 0.5 per cent chromogenic material calculated as carnosine. We include this figure in characterizing our preparations because the purity of any sample of anserine or carnosine is open to question unless the color value is reported with other constants.

It should be noted that anserine preparations may also be contaminated with non-chromogenic material as is obviously the case with the preparations from cat and rabbit muscle. Both products gave low color values but the total nitrogen and melting points indicate the presence of considerable impurities. Treatment with

silver nitrate and barium hydroxide did not materially change the nitrogen content of the preparation from rabbit muscle.

Results

Dog—5.40 kilos of skeletal muscle yielded 7.50 gm. of crude copper salt, m.p. 228–230° (corrected). This was recrystallized from water and dried at 120° for analyses.

Total nitrogen (Kjeldahl)
(*a*) 0.1100 gm. required 13.93 cc. 0.1 N acid
(*b*) 0.1108 " " 13.94 " 0.1 " "
Methylimino group (Herzig and Meyer (12))
0.1106 gm. gave 0.0760 gm. AgI

	Total N	(N)-CH_3
Calculated for $C_{10}H_{16}N_4O_3 \cdot CuO$.	17.53	4.70
Found.	17.61, 17.73	4.34

Color Value—1.5 mg. of salt converted to base and treated with diazotized sulfanilic acid according to Koessler and Hanke's procedure (10) matched 21 mm. of Hunter's (11) dye standard checked against pure histidine.[3] This is 3.5 per cent of the amount of color given by 1.5 mg. of copper carnosine treated similarly. A control experiment with anserine and carnosine in the ratio 100:1 gave the reading calculated for the carnosine plus impurities in the anserine.

Anserine Nitrate—Anserine nitrate was prepared from copper salt after purification with silver nitrate and barium hydroxide. Colorless needles separated from 70 per cent alcohol, m.p. 224° (corrected).

Total nitrogen (Kjeldahl modified to include nitrates (13))
(*a*) 0.0996 gm. required 16.15 cc. 0.1 N acid
(*b*) 0.0994 " " 16.41 " 0.1 " "

Calculated for $C_{10}H_{16}N_4O_3 \cdot HNO_3$.	23.10
Found.	22.87, 23.08

Color value, 1.7 per cent of theory for carnosine

Free Base—Free base was prepared from copper salt after purification with silver nitrate and barium hydroxide. Colorless needles separated from 70 per cent alcohol, m.p. 238° (corrected).

[3] The color standards were prepared and checked by Mr. E. K. Doak.

Total nitrogen
(*a*) 0.1078 gm. required 17.90 cc. 0.1 N acid
(*b*) 0.1150 " " 19.16 " 0.1 " "
Amino nitrogen (Van Slyke)
Barometer reading, 753.6 mm. Hg pressure, at 24.5°
10 mg. gave (*a*) 1.08 cc., (*b*) 1.12 cc. in 2 minutes.
Methylimino group
0.1054 gm. gave 0.0986 gm. AgI

	Total N	NH_2-N	(N)-CH_3
Calculated for $C_{10}H_{16}N_4O_3$.	23.34	5.84	6.24
Found.	23.25, 23.22	5.96, 6.18	5.73

Color value, about 0.5 per cent theory for carnosine

Cat—4.20 kilos of skeletal muscle yielded 5.50 gm. of crude copper salt with typical properties, m.p. 224–225° (corrected). Color value, 2.8 per cent. The copper salt was decomposed and treated with silver nitrate and barium hydroxide, and reconverted to copper salt, m.p. 224–225° (corrected).

Total nitrogen
(*a*) 0.1140 gm. required 14.74 cc. 0.1 N acid
(*b*) 0.1110 " " 14.71 " 0.1 " "
Methylimino group
0.1027 gm. gave 0.0696 gm. AgI

	Total N	(N)-CH_3
Calculated for $C_{10}H_{16}N_4O_3 \cdot CuO$.	17.53	4.70
Found.	18.10, 18.55	4.10

Color value, 0.1 per cent

Rabbit—4.40 kilos of skeletal muscle yielded 9.60 gm. of crude copper salt with characteristic properties, m.p. 227–228° (corrected). Color value, 0.9 per cent. The analyses were unsatisfactory. The salt was decomposed and treated with silver nitrate and barium hydroxide. It was then isolated as (*a*) copper salt, (*b*) free base.

Copper Salt—Copper salt, m.p. 224–226° (corrected), dried at 120° for analyses.

Total nitrogen
(*a*) 0.1096 gm. required 14.06 cc. 0.1 N acid
(*b*) 0.1250 " " 16.08 " 0.1 " "
Methylimino group
0.1398 gm. gave 0.1044 gm. AgI

	Total N	(N)-CH
Calculated for $C_{10}H_{16}N_4O_3 \cdot CuO$.	17.53	4.70
Found.	17.96, 18.01	4.78

Color value, 0.3 per cent

Free Base—The free base had a melting point of 222–227° (corrected). Colorless needles separated from 70 per cent alcohol. They were dried at 120° for analyses.

Total nitrogen
(*a*) 0.1150 gm. required 19.58 cc. 0.1 N acid
(*b*) 0.1164 " " 19.63 " 0.1 " "
Methylimino group
0.1100 gm. gave 0.0979 gm. AgI

	Total N	(N)-CH_3
Calculated for $C_{10}H_{16}N_4O_3$.	23.34	6.25
Found.	23.84, 23.61	5.72

Color value, 0.6 per cent

White Rat—75 animals were skinned, eviscerated, the head, feet, and tail cut off, and the entire carcass ground for extraction. The dressed weight was 8.0 kilos, yield 2.0 gm. of crude copper salt with typical properties, m.p. 224–226° (corrected). The salt was dried at 120° for analyses.

Total nitrogen
(*a*) 0.0916 gm. required 11.33 cc. 0.1 N acid
(*b*) 0.0926 " " 11.52 " 0.1 " "
Methylimino group
0.0851 gm. gave 0.0452 gm. AgI
0.1404 " " 0.0776 " "

	Total N	(N)-CH_3
Calculated for $C_{10}H_{16}N_4O_3 \cdot CuO$.	17.53	4.70
Found.	17.31, 17.42	3.60, 3.53

Color value, 1 per cent

Horse—31 kilos of muscle were obtained from the hind limbs. No trace of anserine was found at any stage of preparation. Carnosine was isolated as the copper salt, in characteristic blue hexagons from silver precipitates.[4]

DISCUSSION

In relation to other nitrogenous extractives, the carnosine content of the skeletal muscles of the horse, ox, and pig (14) is probably second only to creatine. The muscles of these animals contain 0.3 per cent or more of carnosine. The high concentration suggests that it is of importance in muscle metabolism. But, as

[4] Mr. W. Earl Graham assisted in this isolation.

colorimetric determinations indicate a much lower concentration in the muscles of many vertebrates (occasionally it appears to be completely absent) Clifford (15) aptly points out that the universal importance of carnosine cannot be postulated.

The discovery of anserine in muscles of birds, reptiles, and fishes by Ackermann and his coworkers focuses attention on a new compound with properties very similar to carnosine. Our demonstration that muscles of certain mammals contain anserine permits the conclusion that neither anserine nor carnosine is associated exclusively with any particular group of vertebrates. The occur-

TABLE I

Carnosine and Anserine in Certain Muscles

Animal	Isolation procedure		Colorimetric determination of carnosine
	Carnosine	Anserine	
	per cent	*per cent*	*per cent*
Horse	0.18 (16)	None*	0.26–0.48 (14)
Ox	0.26 (17)	None (3)	0.32–0.64 (14, 18)
Cat		0.09*	0.01–0.49 (19)
Dog	Present (1)*	0.10*	
Rabbit	0.22 (20)	0.15*	0.03–0.15 (15, 18)
Chicken	None (3)	0.12 (3)	0.17 (15)
Boa constrictor	Present (4)	None (4)	
Crocodile	" (4)	0.03 (4)	
Conger-eel	None (5)	0.04 (5)	
River eel	0.07 (21)	None (5)	0.37 (15)
Codfish	None (5)	0.03 (5)	None (15)

The figures in parentheses refer to the bibliography.
* This paper.

rence of the two compounds shown in Table I suggests that their functions may be more or less interrelated. In general, muscles high in carnosine are low in anserine and *vice versa*. Accurate comparisons, however, can only be made when more and better data are available. There is some indication that total iminazole content (carnosine plus anserine) may be important for physiological considerations.

SUMMARY

Anserine, a methyl carnosine, has been isolated from skeletal muscles of the dog, cat, rabbit, and white rat and identified by

means of the copper salt or nitrate and free base. No trace of the compound could be found in horse muscle. These observations show that anserine is not generally absent from muscles of mammals as had been concluded from a study of ox muscle. The widespread distribution of iminazoles, either anserine or carnosine, in vertebrate muscle is discussed.

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BIBLIOGRAPHY

1. Krimberg, R., *Biochem. Z.*, **157**, 187 (1925).
2. von Fürth, O., and Schwarz, C., *Biochem. Z.*, **30**, 413 (1911).
3. Ackermann, D., Timpe, O., and Poller, K., *Z. Physiol. Chem.*, **183**, 1 (1929).
4. Hoppe-Seyler, F. A., Linneweh, W., and Linneweh, F., *Z. physiol. Chem.*, **184**, 276 (1929).
5. Ackermann, D., and Hoppe-Seyler, F. A., *Z. physiol. Chem.*, **197**, 135 (1931).
6. Keil, W., *Z. physiol. Chem.*, **187**, 1 (1930).
7. Linneweh, W., Keil, A. W., and Hoppe-Seyler, F. A., *Z. physiol. Chem.*, **183**, 11 (1929).
8. Pyman, F. L., *J. Chem. Soc.*, **133**, 183 (1930).
9. Guggenheim, M., Die biogenen Amine, Berlin, 23 (1924).
10. Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, **39**, 497 (1919).
11. Hunter, G., *Biochem. J.*, **19**, 42 (1925).
12. Herzig, J., and Meyer, H., *Monatsh. Chem.*, **15**, 613 (1894).
13. Methods of analysis of the Association of Official Agricultural Chemists, Washington, 8 (1925).
14. Kuen, F. M., *Biochem. Z.*, **189**, 60 (1927).
15. Clifford, W. M., *Biochem. J.*, **15**, 725 (1921).
16. Smorodinzew, J., *Z. physiol. Chem.*, **87**, 12 (1913).
17. Smorodinzew, J., *Z. physiol. Chem.*, **92**, 214 (1914).
18. Hunter, G., *Biochem. J.*, **16**, 640 (1922).
19. Hunter, G., *Biochem. J.*, **18**, 408 (1924).
20. Yoshimura, K., *Biochem. Z.*, **37**, 477 (1911).
21. Suzuki, U., Joshimura, K., Jamakawa, M., and Irie, Y., *Z. physiol. Chem.*, **62**, 1 (1909).